Camptothecin Overcomes MDR1-mediated Resistance in Human KB Carcinoma Cells

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ABSTRACT

In order to understand the high efficacy of camptothecin derivatives against human colon tumor xenografts in nude mice, we have studied the transport properties of camptothecin derivatives across cellular membranes of MDR1-overexpressing cells. MDR1 overexpression was shown to have little effect on camptothecin cytotoxicity; camptothecin was equally cytotoxic to both the drug-sensitive parental cell line, KB 3-1, and its multidrug-resistant derivative, KB V1. The ability of camptothecin to overcome MDR1-mediated resistance is most likely due to unimpaired accumulation of camptothecin in MDR1 cells as suggested from the following experiments: (a) cytotoxicity of camptothecin against KB V1 cells was not altered by the known MDR1-reversing agent, verapamil; (b) camptothecin was ineffective as compared with vinblastine in competing with [3H]azidopine for photoaffinity labeling of MDR1; (c) camptothecin was equally efficient in trapping cellular topoisomerase I molecules on chromosomal DNA in the form of cleavable complexes in both KB 3-1 and KB V1 cells.

The mechanism by which camptothecin overcomes MDR1-mediated resistance has been further studied using a number of uncharged and charged camptothecin derivatives. In contrast to the uncharged camptothecin derivatives, such as 9-amino-camptothecin and 10,11-methylene-dioxycamptothecin, the charged camptothecin derivative, topotecan, showed reduced cytotoxicity against MDR1-overexpressing KB V1 cells. The reduced cytotoxicity of topotecan in KB V1 cells was due to the overexpression of MDR1 in KB V1 cells since verapamil restored both topotecan accumulation and cytotoxicity. These results suggest that the charge on camptothecin can affect the drug's sensitivity to MDR1. The possible effect of membrane permeability in determining drug selectivity of MDR1 is discussed.

INTRODUCTION

Camptothecin and several analogues exhibit an unprecedented antitumor activity against human colon cancer as well as other cancer types such as lung, breast, and malignant melanoma, carried as xenografts in nude mice (1–3). The unique high efficacy of camptothecin against otherwise refractory solid tumors is not fully understood. The molecular target of camptothecin and its derivatives has been unequivocally identified to be DNA topoisomerase I (4–8), a nuclear enzyme implicated in DNA replication and RNA transcription (9–14). The inhibitory mechanism of camptothecin against topoisomerase I has also been partially elucidated. Camptothecin interefers with the breakage/reunion reaction of topoisomerase I by stabilizing a reversible enzyme-drug-DNA ternary complex, termed the cleavable complex. The formation of the cleavable complex specifically prevents the reunion step of the breakage/union cycle of the topoisomerization reaction (4, 5). The cleavable complex can be denatured with a strong protein denaturant, such as SDS or alkali, to reveal a single-strand break to which a topoisomerase I polypeptide is covalently linked to the 3'-phosphoryl end of the broken DNA strand (4). The cytotoxicity of camptothecin is almost certainly due to the formation of cleavable complexes in cells. The majority of protein-linked DNA breaks observed in cultured mammalian cells treated with camptothecin reflect cleavable complexes formed in cells (5). Recent studies have shown that the formation of cleavable complexes in cells is not, by itself, a lethal event. It is rather the interaction between the cleavable complexes and replication machinery which triggers cell death (15–17). S-phase-specific cytotoxicity of camptothecin may result from such an event (18).

The strong antitumor activity of camptothecin and analogues against human tumors could be due either to a number of factors that affect the ability of the drugs to accumulate in cancer cells or to the level of potentially lethal DNA damage induced by camptothecin. The higher levels of topoisomerase I in surgical specimens of human colon tumors as compared with normal mucosa (1, 2) could lead to increased lethal DNA damage in tumor cells. Recent studies have suggested that topoisomerase I levels may be influenced by oncogene expression and by the agents that affect the mitogenic signal transduction pathway (19, 20). However, the increased topoisomerase I level is probably not solely responsible for the observed high efficacy of camptothecin derivatives, since a parallel increase in the topoisomerase II level in colon tumors was also observed. Despite this increase in topoisomerase II levels, topoisomerase II-active antitumor drugs (e.g., VP-16 and Adriamycin) are generally ineffective against human colon tumors in the nude mouse system (1–3).

Human colon tumors have also been shown to express relatively high levels of the MDR1 gene product (21), which, in cultured cells, has been shown to be responsible for multidrug resistance by restricting accumulation of certain drugs in cells (22). The gene expression seems to be closely related to local tumor aggressiveness and lymph node invasiveness (23). The strong antitumor activity of camptothecin against human colon tumor xenografts in nude mice led us to investigate the possibility that camptothecin may overcome MDR1-mediated resistance. This premise was confirmed by preliminary studies (3). Current study compares several charged and uncharged camptothecin analogues, with various degrees of water solubility.

MATERIALS AND METHODS

Cell Lines. The drug-sensitive human epidermoid cell line KB 3-1 and its vinblastine-selected MDR variant KB V1 were kindly provided to us by Dr. Michael M. Gottesman (National Cancer Institute). Cell line KB V1 was isolated from KB 3-1 by stepwise selection with

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2 To whom requests for reprints should be addressed, at Department of Biological Chemistry, Johns Hopkins School of Medicine, N. Wolfe St., Baltimore, MD 21205.
Table 1 Camptothecin is equally cytotoxic to human KB 3-1 and KB VI cells

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC₅₀ for drug-sensitive KB 3-1 cells (nM)</th>
<th>Relative resistance*</th>
</tr>
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<tbody>
<tr>
<td>CPT</td>
<td>7.1 ± 0.4</td>
<td>1.2x</td>
</tr>
<tr>
<td>9A-CPT</td>
<td>7.3 ± 0.2</td>
<td>1.7x</td>
</tr>
<tr>
<td>10H-CPT</td>
<td>32 ± 2</td>
<td>1.9x</td>
</tr>
<tr>
<td>TPT</td>
<td>25 ± 0.4</td>
<td>8.8x</td>
</tr>
<tr>
<td>10,11-CPT</td>
<td>1.0 ± 0.1</td>
<td>1.3x</td>
</tr>
<tr>
<td>9N-10,11-CPT</td>
<td>15 ± 0.4</td>
<td>1.7x</td>
</tr>
</tbody>
</table>

* Cytotoxicity of camptothecin analogues against KB 3-1 and KB VI cells was determined as described in “Materials and Methods.”

**Relative resistance = IC₅₀ for KB VI cells/IC₅₀ for KB 3-1 cells.


increasing vinblastine (24). KB V1 cells are 420 times more resistant to Adriamycin and 210 times more resistant to vinblastine than KB 3-1 cells (25).

Chemicals and Drugs. Camptothecin and its derivatives 9-amino-camptothecin, 10,11-methylenedioxy-camptothecin, 10-hydroxy-camptothecin, 9-nitro-10,11-methylenedioxy-camptothecin, and topotecan (hycamptamine) were synthesized as described previously (26-28). Except for 10,11-methylenedioxy-camptothecin, which was a racemic mixture, all other camptothecin derivatives are 20(S) stereoisomers. Also, except for 10-hydroxy-camptothecin, which was in its sodium salt form, all other camptothecin derivatives were in their lactone form. All camptothecin derivatives were dissolved in dimethyl sulfoxide at 20 mg/ml and stored at -20°C. Vinblastine and verapamil were purchased from Sigma Chemical Co. [3H]Azidopine (40 Ci/mmol) and 125I-protein A (30 mCi/mg) were obtained from Amersham Corp.

Cytotoxicity Assays. Multidrug-resistant KB VI cells were cultured in the absence of drugs for at least two doublings prior to cytotoxicity assays. As described previously (29), 2-4 x 10⁴ KB 3-1 or VI cells were plated in 6-well plates and exposed to varying concentrations of drugs in the absence or presence of 10 µM verapamil for 4 days before cell numbers were determined using a Coulter Counter (Coulter Electronics, Inc.).

![Chemical structures of some camptothecin analogues](image)

Fig. 1. Chemical structures of some camptothecin analogues.
can be determined by immunoblotting (see “Materials and Methods”). Equivalent numbers of KB 3-1 (A) and KB VI cells (B) were treated with 45 μM camptothecin (C) or topotecan (D) for 0, 10, 20, 30, 40, and 90 min. The amounts of untrapped cellular topoisomerase I molecules were determined by immunoblotting using rabbit antisera against human DNA topoisomerase I. Autoradiographs, region representing the M, 100,000 topoisomerase I. The graphs displayed below were constructed from densitometric tracings of the autoradiographs. Percentage of untrapped topo-1 is defined as 100 × (drug-treated)/control.

Table 2 Verapamil cotreatment with topotecan increased the level of cleavable complexes in KB VI cells

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Camptothecin</th>
<th>Topotecan</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>(-) VR &lt;sup&gt;a&lt;/sup&gt;</td>
<td>(+) VR &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>240</td>
<td>55</td>
<td>44</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of untrapped topoisomerase I = 100 × (drug-treated)/control.

<sup>b</sup> VR, 10 μM verapamil.

ylenedioxy-camptothecin (for structures, see Fig. 1), were also tested and shown to be about equally cytotoxic against both cell lines (Table 1). This is in contrast to the more than 200-fold resistance of KB V1 cells to Adriamycin and vinblastine (25). Several lines of evidence support the notion that the ability of camptothecin to overcome the MDR1-mediated resistance of KB V1 cells is due to its unimpaired accumulation in MDR1 cells. First, the cellular level of topoisomerase I, as measured by immunoblotting with topoisomerase I antisera, was the same in both KB 3-1 cells and KB V1 cells (Fig. 2, 0-min samples). Second, the accumulation of camptothecin in cells as indicated by the level of cellular cleavable complexes, measured by immunoblotting of untrapped cellular topoisomerase I, was about the same in both KB 3-1 cells and KB V1 cells (Fig. 2, 0–90-min samples). Third, the MDR1-reversing agent verapamil (10 μM) had no effect on either camptothecin accumulation (Table 2) or cytotoxicity in KB V1 cells (Fig. 3). In contrast, under the same conditions, Adriamycin cytotoxicity against KB V1 cells was largely reversed by verapamil cotreatment (data not shown).

Topotecan, a Charged Derivative of Camptothecin, Showed Reduced Accumulation and Cytotoxicity in KB V1 Cells. Topotecan is a charged derivative of 10-hydroxy-20S-camptothecin (Fig. 1). The dimethyl-amino-methyl substitution at the position 9 carbon confers a positive charge on the camptothecin molecule at neutral pH. Topotecan, in contrast to other non-charged camptothecin derivatives, showed much reduced cytotoxicity against KB V1 cells (IC<sub>50</sub> about 220 nM) than KB 3-1 cells (IC<sub>50</sub> about 25 nM) (Table 1 and Fig. 4). The reduced topotecan cytotoxicity against KB V1 cells was accompanied by a reduction in the amounts of drug-trapped cleavable complexes as measured by immunoblotting (Fig. 2). While about 40% of total cellular topoisomerase I molecules were trapped onto chromosomal DNA as cleavable complexes by topotecan in KB 3-1 cells within 10 min of drug treatment, only about 10% of cellular topoisomerase I molecules were trapped onto chromosomal DNA in KB V1 cells even after 90 min of drug treatment (Fig. 2). This is in contrast to the rapid trapping of about 50% of the total cellular topoisomerase I molecules onto chromosomal DNA by camptothecin in both KB V1 and KB 3-1 cells within 10 min of drug treatment (Fig. 2). These results suggested that the reduced cytotoxicity of topotecan against KB V1 cells was most likely due to the reduced accumulation of topotecan in KB V1 cells.

The hypothesis that reduced topotecan accumulation accounts for reduced topotecan cytotoxicity against KB V1 cells was further supported by an experiment using the MDR1-reversing agent verapamil. As shown in Fig. 3, verapamil (10 μM) was able to markedly sensitize KB V1 cells against topotecan. In contrast, verapamil under the same conditions had no effect on camptothecin cytotoxicity against KB V1 cells (Fig. 3). In addition, a good correlation between sensitizing of KB V1 cells to topotecan and restoring cellular topotecan accumulation by verapamil was demonstrated (Table 2).

Camptothecin Did Not Compete with [3H]Azidopine for Photoaffinity Labeling of MDR1. The interaction between camptothecin and MDR1 was also examined through the ability of camptothecin to compete with [3H]azidopine in photoaffinity labeling of MDR-1 prepared from KB V1 cells. As shown in Fig. 5, camptothecin (Fig. 5, Lanes E–G) did not compete with [3H]azidopine even at a molar ratio of 2500:1, whereas vinblastine (Fig. 5, Lanes B–D) nearly completely prevented [3H]-azidopine photoaffinity labeling of the MDR1 at the lowest vinblastine concentration (molar ratio, 100:1). Topotecan, which is also a weak competitor as compared with vinblastine,
Fig. 3. Verapamil can restore the sensitivity of MDR1-overexpressing KB VI cells to topotecan. Cytotoxicity of topotecan (A) and camptothecin (B) against KB VI cells in the absence and presence of 10 nM verapamil was determined. Points, mean of two independent determinations.

Fig. 4. Topotecan, a charged derivative of camptothecin, is less cytotoxic against KB VI than KB 3-1 cells. Cytotoxicity of topotecan (A) and camptothecin (B) were determined as described in "Materials and Methods." Points, mean of two independent determinations.

Fig. 5. Interaction between MDR1 and camptothecin as determined by [3H]azidopine photoaffinity labeling of MDR1-containing membrane from KB VI cells. Membranes (50 μg of membrane proteins for each determination) prepared from MDR1-overexpressing KB VI cells were photolabeled using 50 μM [3H]azidopine in a final volume of 50 μl. Photoaffinity-labeled membranes were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis. Lane A, [3H]azidopine-labeled membranes; Lanes B–J, [3H]azidopine-labeled membranes in the presence of 2500- or 500- or 100-fold molar excess of the following drugs: vinblastine (Lanes B–D), camptothecin (Lanes E–G), and topotecan (Lanes H–J).

was demonstrated to be at least 5-fold more potent than camptothecin in competing with [3H]azidopine for photoaffinity labeling of MDR1 (Fig. 5, Lanes H–J).

DISCUSSION

Our results show that camptothecin and a number of its uncharged analogues can overcome MDR1-mediated resistance. The fact that camptothecin can trap equal amounts of topoisomerase I-cleavable complexes in both KB 3-1 and KB VI cells suggests that camptothecin accumulation is unimpaired in MDR1-overexpressing KB VI cells. Overexpression of MDR1 has been well documented to reduce intracellular accumulation of a number of structurally unrelated drugs, including vinblastine and Adriamycin (22). The reduced accumulation of these drugs in MDR cells is presumed to be due to the action of membrane-bound MDR1, which acts as an energy-dependent drug efflux pump (33, 34). The drug selectivity of MDR1 is still not understood. The apparent insensitivity of camptothecin to MDR1 may therefore provide an important clue to the mechanism of drug selectivity by MDR1.

Camptothecin might conceivably overcome MDR1-mediated resistance by either of the following two mechanisms. One possible mechanism is that camptothecin, being a planar hydrophobic compound, can rapidly diffuse through cellular membrane, and the relatively weaker drug efflux pump (MDR1) is unable to affect the cellular accumulation of camptothecin. The second possible mechanism is that camptothecin is not recognized by MDR1. The fact that the charged camptothecin derivative, topotecan, is sensitive to the overexpression of MDR1 appears to support the first possibility. Topotecan is expected to have a much reduced membrane permeability due to the charge. The slower rate of passive diffusion of topotecan when
compared to the relatively faster rate of MDR1-mediated drug efflux may therefore lead to reduced topotecan accumulation in MDR cells. However, one cannot rule out the second possibility, since recognition by MDR1 may require a positive charge, which is present in topotecan and absent in other camptothecin derivatives. Indeed, studies of a number of MDR-reversing agents have suggested the importance of a positive charge (35).

The [3H]azidopine photoaffinity labeling experiment, on the other hand, seem to support the second mechanism. The simplest explanation for the lack of competition of camptothecin is that MDR1 does not recognize camptothecin. However, the interpretation of the azidopine photolabeling experiment may be quite complex. Perturbation of the cellular membrane may also be an important contributing factor in the competition of drugs with azidopine for MDR1 binding. It is possible that camptothecin does not significantly perturb the cellular membrane and therefore does not compete effectively with azidopine for MDR1 binding. If this is the case, drug selectivity of MDR1 could be determined at least in part by drug-membrane interaction.

Regardless of the drug selectivity of MDR1, rapid passive diffusion of camptothecin is probably sufficient to explain the ability of camptothecin to bypass MDR1-mediated resistance. Our results with camptothecin therefore might provide an explanation for the strong antitumor activity of camptothecin against human colon tumor xenografts in nude mice (1). It seems plausible that MDR1 overexpression in human colon tumors may be a significant contributing factor to the observed intrinsic resistance of these tumors to chemotherapy using other anticancer agents (36). Camptothecin and some of its analogues, being able to rapidly diffuse into cells, are effective against colon tumors. However, it is also possible that drug transport and hence drug accumulation in human colon tumors may be restricted due to factors other than overexpression of MDR1. Further studies are necessary to establish whether MDR1 overexpression is responsible for the observed intrinsic multidrug resistance of human colon tumors.

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